IN THE UNITED STATES PATENT OFFICE

Application Serial No. 07/675,908

Filed: July 3, 1991

Applicants: Dr. Rudolf Falk
Dr. Samuel S. Asculai
(Now assigned to
Hyal Pharmaceutical Corporation)

MAIL ROOM 14 OCT 16 1996 PAT. & TRADEMARK OFF.

Title: THE USE OF HYALURONIC ACID OR ITS DERIVATIVES TO ENHANCE DELIVERY OF ANTINEOPLASTIC AGENTS

Inventors: Dr. Rudolf Falk,
Dr. Samuel S. Asculai

Examiner: Dr. Jacqueline Krikorian Ph.D. (formerly Dr. Stephen Martin, Ph.D.)

Group Art Unit: 1806 Extended Due Date: September 5, 1996

The Commissioner of Patents
UNITED STATES PATENT OFFICE
2011 Jefferson Davis Highway
Crystal Plaza 2, Room 1B03
Arlington, Virginia
U.S.A. 22202

DECLARATION OF DR. ADRIAN RICHARD MOORE under § 1.132

I, ADRIAN RICHARD MOORE, Ph.D., make oath and say as follows:

1. (a) I am an Honorary Lecturer and Senior Research Fellow for the Department of Experimental Pathology, William Harvey Research Institute, Charterhouse Square, London, England. A copy of my Curriculum Vitae is attached as *Exhibit 1* to this my Declaration. I have worked with hyaluronan over a period of approximately 10 years. Initial

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work was carried out on behalf of Fidia in the area of degenerative joint disease. Subsequently, I have been working on behalf of Hyal Pharmaceutical Corporation to investigate the ability of hyaluronan to target drugs to sites of pathology. This was for me, an expected use of hyaluronan and indeed initial experiments had to be repeated because the importance of administering a mixture of drug and hyaluronan (rather than each agent separately) was not obvious to me at the time.

(b) The Department of Experimental Pathology is in receipt of a grant from Hyal Research Foundation. I would not, however, allow this financial interest to cloud my professional judgement and responsibilities in preparation of a declaration for any person.

2. I have been asked to review the teachings of International Publication No. WO 91/04058 which I was advised by Ivor Hughes, counsel for Hyal Pharmaceutical Corporation, entered the national phase in the United States Patent Office as Application No. 07/675,908. This patent application relates to dosage amounts of compositions which combine between 10 and 1,000 mg or more of Hyaluronic Acid in the dosage together with an effective amount of a medicine/therapeutic agent which may be suitable to treat a disease or condition and which Hyaluronic Acid alters the distribution and performance in the human body of the medicines and produces an unusual targeting of the medicines and therapeutic agents for underperfused tissue and/or pathological tissue. The amounts of Hyaluronic Acid may vary from 10 mg/70 kg person in the dosage amount to 1000 mg or more/70 kg person in the dosage amount with optimal doses tending to range between 50 and 350 mg/70 kg individual. While the patent specifies that the Hyaluronic Acid can obviously be administered, for example in excess of 3000 mg/70 kg person, because there appears to be

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no toxicity, this does not imply to any person skilled in the art that any amount could be administered without ill effect. I believe that Dr. Falk sometimes describes minor fluid retention in his patients receiving HA, but irrespective of that, "heroic" doses of any material will eventually be toxic. It would obviously be prudent to persons skilled in the art to use the minimum dose of Hyaluronic Acid for efficacy. Another danger of "heroic" doses is that however low the impurities may be, if the dose were large enough, they could cause problems, e.g., sensitization. However, persons skilled in the art would have no difficulties in choosing the appropriate dosage amount from the disclosure in the Application.

3. The molecular weights of Hyaluronic Acid which could be used are discussed at page 29, 30, 31 and 32 and provide molecular weights of Hyaluronic Acid ranging between 150,000 daltons and less than 750,000 daltons. This range to me seems to be an entirely reasonable range given that with respect to very low molecular weights, they would not be included in this range. Particularly, I am aware of West and Kumer (Exp. Cell. Res. 1989, 183: 179 to 196) which describes low molecular weight (3 to 16 disaccharide units), HA derivatives which stimulate angiogenesis *in vivo* and endothelial cell proliferation *in vitro*. A high molecular weight fraction (greater than 16 disaccharide units) did not share these properties. It is therefore clear to me that the use of dosage amounts comprising between 10 mg to 1000 mg/70 kg person of the Hyaluronic Acid or salts thereof and which has a molecular weight between about 150,000 daltons and 750,000 daltons, together with a medicine or therapeutic agent suitable for use in treating a disease/condition would alter the medicine's distribution and performance in the human and body and produce an unusual targeting for underperfused tissue and/or pathological tissue to be treated. It is in this regard the invention relates and not to a mere

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combination of Hyaluronic Acid and medicines. It is also in respect of methods of treatment arising from the use of these dosages that the invention arises. By administering these dosages as taught in the application, totally unexpected results occur such as with persons who have been unresponsive to conventional treatment for cancer who respond positively and the patient goes into remission and the patient's life is extended and the patient's quality of life is also enhanced. The patient does not suffer pain. See for example the cases described beginning at page 36.

4. Given my experience with respect to Hyaluronic Acid prior to 1989, I would not have expected these results from the dosage forms and the administration of these dosage forms, nor would persons skilled in the art have expected these results. The results are totally unexpected as will be evidenced when I discuss the prior art references referred to me for my opinion in this my Affidavit.

5. I have also been asked to comment in respect of other matters. For example, calcium channel blockers are generally used to treat an angina and essential hypertension. The application provides that the tests were carried out at the bottom of page 33, line 34 to line 37, (Applicant has combined Hyaluronic Acid and sodium hyaluronate with medicinal and/or therapeutic agents for the treatment of conditions and diseases with totally unexpected results, for example), thereafter listing conditions and diseases and chemicals and drugs, one of which is subparagraph 16, hypertension, cardiac insufficiency, using calcium channel blockers. At page 35, line 23, nifedipine, a calcium channel blocker is listed.

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6. Discussions with respect to the mechanism are provided. Since that time, more is understood, and the targeting of systemically administered Hyaluronic Acid (HA) to the sites of trauma is now thought to be due to an overexpression of HA receptors at those sites, i.e. an overexpression of HA receptors which remain unfilled at the sites of any condition or disease, i.e. malignant tumour, for example, at the site.

7. As an expert in the field of Hyaluronic Acid reading the said application WO 91/04058, I would have thought that it was self-evident that any salts of HA which are toxic or not pharmaceutically acceptable would be excluded by persons skilled in the art reading the application. In my opinion, the terms "salts", "non-toxic salts" and "pharmaceutically acceptable salts" are interchangeable having regard to the teachings of the application.

8. Having regard to molecular weights and viscosity, and having read the application, I have concluded that persons skilled in the art would have no difficulties in formulating suitable dosage amounts of the invention reading this application. Particularly, persons preparing the dosage amounts with the medicines would dilute the Hyaluronic Acid to form the dosages for incorporation of the medicine/therapeutic agents. Persons skilled in the art would know that administration of the dosages would be easier where the dosages are less viscous. This is clear from the teachings at page 33, line 29 to 31 of WO 91/04058 which provides "where high molecular weight Hyaluronic Acid (or salts or other forms thereof) is used, it must be diluted to permit administration and ensure no intramuscular coagulation." Thus whether or not viscosity is mentioned, persons skilled in the art reading the application would not use highly viscous formulations. In fact, molecular weights of HA between 150,000 daltons

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and 750,000 daltons are not very viscous and the reader will note the use of a 2% solution discussed at page 29, line 35 of the sodium hyaluronate which has a mean average molecular weight of about 225,000. Persons skilled in the art, in my opinion, would have no trouble preparing suitable dosage forms.

9. Additionally, where in excess of 200 mg of the form of Hyaluronic Acid is used in the dosage amount with, for example NSAIDS such as indomethacin, no major toxic side effects occur, such as gastro intestinal distress, neurological abnormalities, depression, etc. even at elevated amounts of indomethacin. Thus, where it is desired to use an NSAID, excess amounts of accepted amounts of the NSAID may be used to achieve desired results and enable the patients to take more of the medicine and not have any side effects. I have reviewed the application in regards to examples where this occurs and refer the reader's attention to Case VIII at page 45, line 25 wherein the patient received systemically indomethacin 300 mg daily with 300 mg Hyaluronic Acid, there does not appear to be any problem, and in fact the tumor mass decreased significantly in size and it could not be distinctly identified on this examination, (page 45, lines 34 to 36). Additionally, at page 53, Case XVIII, the patient was given Hyaluronic Acid up to 300 mg daily with indomethacin, 300 mg daily, with what appears no side effects. The alkaline phosphotase of the patient decreased, and the patient's liver appeared functioning better (see page 53, lines 1 to 6). In Case XIX, referred to at page 53, the patient was given 300 mg of indomethacin, an excessive dose, and caused this patient, even though the patient took hyaluronic acid 300 mg, heartburn. This dosage of indomethacin was reduced to 100 mg which was still an excessive dose (more than a normal dose), and the patient was able to accept this dosage

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without any side effects. The patient improved dramatically after the first five days of therapy.

Although I am not a physician, I believe it is generally recognized that patients will differ in their response to medication. The ability to use elevated doses of NSAIDs when given with HA is therefore likely to vary between patients. However, in a normal gastrointestinal tract HA would be expected to shield the tissue from the NSAID therefore allowing their dose to be increased without major toxic side effects. There may nonetheless be some instances where administration of HA with an NSAID may be detrimental. For example, patients with gastrointestinal damage may over express HA receptors at the sites of their lesions which could therefore be targeted by the NSAID to increase the damage. The physicians will be able to monitor this situation.

10. It is thus clear to me that International Publication No. WO 91/04058 clearly teaches persons skilled in the art to use the invention. I reach this conclusion having regard to the teachings in the application as a whole, including the cases exemplified in the application, Cases I to XL, the further tests beginning at page 67, and the anecdotal tests referred to in the application such as at the bottom of page 25 and starting at the bottom of page 33 to page 35 and the additional tests illustrated after Case XV beginning at page 51, line 26 to page 52, line 4.

11. Having regard to the application as a whole, it is clear that persons skilled in the art in reading the application would be able to prepare dosages suitable for treating the patients with the medicines and/or therapeutic agents contemplated for such treatments and that such dosages enhance the distribution and performance of the medicine in the human body by

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the use of the form of Hyaluronic Acid to produce an unusual targeting for underperfused tissue and/or pathological tissue which targeting was not previously known.

12. Thus is nowhere taught in the prior art, and in fact, based on my experience is completely foreign to the teachings and knowledge in the prior art prior to the filing of the priority application which I am advised by the said Ivor Hughes was filed September 21, 1989 in Canada. I myself, found the invention to be totally unexpected giving totally unexpected results, and in fact, much of my research thereafter has been based upon the earlier findings of Drs. Falk and Asculai in International Publication No. WO 91/04058.

13. From a review of literature prior to 1989, I am aware of the suggestion that Hyaluronic Acid and medicine could be combined and put into the eye to provide a depot effect, or that a depot effect could be created in the treatment of the intraarticular cavity in joints by the use of high molecular weight Hyaluronic Acid as suggested by Balazs (molecular weights in excess of 2,000,000) so that medicines leech (leak) from the formulation and the medicine is absorbed once leeched (leaked) from the formulation onto, for example, the surface of the eye. It is in this regard that United States Patent No. 4,736,024, Della Valle, teaches. This patent teaches topical administration of Hyaluronic Acid with pharmacologically active substances, the advantage being a better bioavailability of the active substances compared to that obtainable with known pharmaceutical formulations in saline and to uses of HA as a vehicle in association with a pharmacological substance to provide an improved drug delivery system by depoting the substance and providing a retard effect. The prime focus of the patent is the ophthalmic use of the invention, although by analogy,

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the document does suggest use in dermatological and other areas (see column 4, lines 62, to column 5, line 6). However, it is by analogy that the treatments using Hyaluronic Acid and medicines is suggested, and thus would be accomplished in the manner as described with the eye, and particularly found at column 1, lines 46 to 53, "when the medicaments are administered in the form of concentrated solution with elastic-viscose characteristics or in solid form, it is possible to obtain films on the corneal epithelium which are homogeneous, stable, perfectly transparent, and which adhere well, guaranteeing prolonged bioavailability of the drug, thereby forming excellent preparations with a retard effect. See also column 2, lines 41 to 51. The dosages given for Hyaluronic Acid are merely drops containing a fraction of a milligram of Hyaluronic Acid. This is clear from the references at column 27, line 57, (micro syringe (10 mcl)); column 29, line 30 (micro syringe (10 µl)); column 31, line 52 (3 drops); column 33, line 24 (2 drops). It is therefore clear to persons skilled in the art that the teachings relate to the application of the dosage to the cornea from which film created on the cornea, the medicine leeches out and is absorbed. This is clear from the statements made at column 30, lines 65 to 68 "Trans-corneal penetration of pilocarpine seems therefore to depend on the capacity of Hyaluronic Acid to vehicle the drug forming a homogeneous and stable film on the cornea." To persons skilled in the art, the description is that the medicine leeches from the formulation and is absorbed, and it is only when the medicine leeches from the film that it can be absorbed.

14. United States Patent No. 4,808,576 to Schultz states that HA will be carried to traumatized tissue by the mammal's natural processes if applied at a site remote from the traumatized tissue. The authors state "it was expected that molecules of such a molecular weight (greater than 1,000,000 daltons)

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would be too large to transfer to the remote site of trauma. The significance of their purported discovery lies in the use of conventional routes of administration, i.e. intra muscular, subcutaneous and topical.

15. For topical use, Schultz specifies the use of a transdermal carrier, giving a number of examples including benzyl alcohol, polyethylene glycol, DMSO and sodium salicylate. However, the HA being the therapeutic agent will not be transported unless the sodium hyaluronate is accompanied by a transdermal carrier. The topical application of the sodium hyaluronate without a transdermal carrier was ineffective. (See column 12, lines 14 to 17.) In all typical examples, DMSO was the carrier. DMSO is the most potent carrier which takes topically applied medications and takes it systemically. DMSO also provides analgesic activity. It is therefore unclear what is providing the benefits in Schultz. The other examples are inconclusive.

16. In Balazs, the article entitled "Hyaluronic Acid: It's Structure and Use", there is no evidence in the literature that any Hyaluronic Acid - in any solvent or with any added carrier will penetrate deeper than the crevices between the desquamating cells. This document teaches to persons skilled in the art that HA sits on the skin but does not penetrate. It does not imply, teach or suggest the use of HA to transport or deliver any agent.

17. U.K. Patent No. 769,287 to Seifter relates to the use of partially depolymerized HA (PDHA) as a spreading and lipemia clearing agent. Undepolymerized HA has no useful spreading effect. Because of the spreading nature of the PDHA, any medicines given are diluted. Whatever the mechanism, the PDHA did not target any disease sites or sites of conditions such as inflammation. Seifter provides a completely

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opposed teaching with their use of PDHA. It spreads through the intercellular matrix taking the medicine with it and consequently works in a similar manner as hyaluronidase without the disadvantages thereof and thus interferes with the intercellular matrix.

18. I am aware of, and am in agreement with, the explanation offered by Dr. Fraser in his Declaration filed in respect of this U.S. Application for the spreading effects of PDHA. This is distinct from the targeting of Drs. Falk and Asculai's invention.

19. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements will jeopardize the validity of the application and any patent issuing thereon.

EXECUTED this 30th day

of August, 1996.

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EXHIBIT 1

CURRICULUM VITAE

of

ADRIAN RICHARD MOORE

Department of Experimental Pathology
William Harvey Research Institute
Charterhouse Sqare
London EC1M 6BQ

Submitted to Ivor M. Hughes, Hughes Etigson, 175 Commerce Valley Drive West, Suite 200, Thornhill, Ontario, Canada L3T 7P6

August 1996

Personal details

Date of birth	1 February 1960.
Nationality	British.
Marital status	Married with two sons.
Home address	101 Windsor Road, Ilford, Essex IG1 1HG.
Home telephone	0181-514 6388.
Work address	Department of Experimental Pathology, The Medical College of St. Bartholomew's Hospital, Charterhouse Square, London EC1M 6BQ.
Work telephone	0171-982 6030.
Work fax	0171-982 6095
E-mail	a.r.moore@qmw.ac.uk

Academic background

	Council for National Academic awards.
1991	Awarded PhD for thesis titled *The Inflammatory Response as a Modulator of Cartilage Breakdown.*
	Open University.
1987	Genetics (Grade 2). Biology, Brain and Behaviour (Grade 2).
1986	Biology, Form and Function (Distinction).
1985	Science Foundation Course (Distinction).
	Institute of Medical Laboratory Sciences.
1983	Special Examination (Cellular Pathology)
	Technicians Education Council
1981	HTEC Medical Laboratory Sciences (Applied Histology). Merits in Chemistry, Biochemistry and Cell Physiology.

	Paddington College, London
1979	Induction Course in Medical Laboratory Sciences
	De Staflord School, Caterham, Surrey
1978	'A' levels Biology (C), Chemistry (B), Mathematics (C).

Professional organisations

Associate of the Institute of Biology.
Member of Research Defence Society

Prizes and distinctions

1988 finalist for the President's Prize (Pathology Section), Royal Society of Medicine.

Grants held

1996	Hyal Research Foundation (joint award)	£212149
1995	Ono Pharmaceutical Co. (joint award)	£432741
1994	Wolfson Foundation	£2000
1993	The Arthritis and Rheumatism Council	£4155

Patents

PCT/GB95/02249	Pharmacological control of inflammation
Pending	Treatment of cancers and other tumours

Post graduate teaching experience (1988-date)

Lectures

East London Rheumatology Group.
Rheumatology seminars, Royal Free Hospital.
The London Inflammation Group.
Pharmacology seminars, Royal College of Surgeons.
Chiropody seminars, St. Bartholomew's Hospital.

Co-supervisor of four Ph.D projects

1) The role of stress proteins in joint destruction.	D. Willis
2) The effects of sex steroids on the inflammatory response.	J. Da Silva
3) Wound Healing	N. Brown
4) Oral tolerance	P. Phipps

Undergraduate teaching experience (1988-date)

Lectures

B.Med.Sci.	Medical College, St. Bartholomew's Hospital.
B.Sc (Exp. Pathology)	Medical College. St. Bartholomew's Hospital.
B.Sc (Chiropody)	Central London Polytechnic.

Project supervision

B.Sc (Exp. Pathology)	Medical College. St. Bartholomew's Hospital.
B.Sc (Biochemistry)	Medical College. St. Bartholomew's Hospital.
Sandwich students	North London Polytechnic, North East London Polytechnic, Manchester Polytechnic.

Recent invited presentations to international symposia

1996	ISRT, Paris
	Fourth Hyal Research Review, Paris
1995	Third Hyal Research Review, Nyons
	INWIN, Geneva
	7th International Seminar on the Treatment oif Rheumatic Diseases, Israel
1994	IRA, USA
	Second Hyal Research Review, Toronto.
	The Charterhouse Conference & Communications Meeting on Bone Cartilage & Synovium, London
1993	First Canadian Workshop for Rheumatology, Florida
	ILAR, Barcelona
	German Rheumatology Society, Garmisch

Work experience

Address: William Harvey Research Institute. London.

Department: Experimental Pathology.

Period: 1994-date

Post: Honorary Lecturer and Senior Research Fellow

Duties: Chairman of departmental finance committee. Deputy Project licence holders. Animals (Scientific Procedures) Act 1986. Head of departmental contract research. Supervision of post graduate students. Examiner and project supervisor for B.Med.Sci. Currently responsible for 1 post doc. 2 Ph.D students. 1 Research Assistant and 1 Senior Technician.

Period: 1991-1994

Position: Post-Doctoral Research Assistant

Duties: Development of novel assay systems with the specific aim of assessing and predicting joint damage in arthritic conditions.

Period: 1988-1991.

Position: Ph.D student.

Duties: *In vitro* cell culture work with macrophages, fibroblasts, osteoclasts, cartilage and neutrophil polymorphs. *In vivo* models including pleurisy, air pouch, adjuvant arthritis, experimental allergic encephalomyelitis and cartilage implantations. Completions of Ph.D thesis. Computer programming for special data handling requirements. Departmental safety officer.

Period: 1982-1988.

Address: St. Bartholomew's Hospital, London.

Department: Histopathology.

Position: MLSO and Senior MLSO.

Duties: Immunological and histopathological service. Specialised techniques included resin work, autoradiography, immunocytochemistry, enzyme histochemistry, morphometry, microdensitometry, spectrophotometry, chemiluminescence and liquid scintillation counting. Radiological Protection Officer. Responsible for managing laboratory finance and maintaining laboratories and apparatus.

Period:	1980-1981.
Department:	Immunodiagnosis.
Position:	MLSO.
Duties:	Immunological screens including immunofluorescence. haemagglutination and radial immunodiffusion.
Period:	1978-1980.
Department:	Histopathology.
Positions:	Junior 'A' MLSO and Junior 'B' MLSO.
Duties:	Production of paraffin wax sections of human pathological material. Frozen sections of urgent biopsy material.

Outside interests

Photography. gardening and DIY.

Peer-reviewed publications

1996

Willis D, Moore AR, Frederik R, Willooughby DA. *Heme oxygenase: A novel target for the modulation of the inflammatory response.* Nature Medicine 2,87-90.

Gowland G, Moore AR, Willis D, Willoughby DA. *Marked enhanced efficacy of cyclosporin when combined with hyaluronan. Evidence from two T-cell-mediated models.* Clin. Drug. Invest. 11. 245-250.

Seed MP, Trancart MM, Moore AR, Azam Z, Prigent D, Willoughby DA. *Metalloproteinase (MMP) regulation and cartilage matrix loss during granulomatous inflammation in elastase deficient Chediak-Higashi mice.* Clin. Rheumatol. In press.

1995

Moore AR, Willis D, Gilroiy D, Tomlinson A, Appleton I, Willoughby DA. *Cyclooxygenase in rat pleural hypersensitivity reactions.* Adv. Prostaglandin, Thromboxane & Leukotriene Research 23, 349-351.

Moore AR, Willoughhby DA. *The role of cAMP regulation in controlling inflammation.* Clin. Exp. Immunol. 101, 387-389.

Moore AR, Gowland G, Appleboam A, Frederik R, Willoughby DA. *Eleastase-alpha-2-macroglobulin complexes and cartilage degradation.* Inflammation Research 44(S3), S254.

Moore AR, Willoughby DA. *Hyaluronan as a drug delivery system for diclofenac: a hypothesis for mode of action.* Int. J. Tissue React. 17, 153-156.

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Moore AR, Gowland G, Willis D, Willoughby DA. *Evaluation of cyclosporin in hyluronan in two models of immune-driven chronic inflammation.* Royal Soc. Med. Round Table Series 40, 106-109.

Willis D, Moore AR, Gowland G, Willoughby DA. *Polyarthritis in the rat: effects of tolerance and sensitisation to the bacterial extract OM-89 with a possible mode of action.* Br. J. Rheumatol. 34, 1135-1138.

Willoughby DA, Moore AR, Papworth J, Seed MP. *Additional comments on non-steroidal drugs and topical analgesia.* Pain 64, 205.

Willis D, Moore AR, Gowland G, Willoughby DA. *Is tolerance induction a possible mode of action of OM-89?* Drugs Under Experimental Clinical Research 11, 207-210.

Appleton I, Moore AR, Willoughby DA. *Vascular endothelial growth factor/vascular permeability factor. effects of hyaluronic acid: comparison with other permeability factors.* Royal Soc. Med. Round Table Series 40, 98-103.

Klemm P, Warner TD, Willis D, Moore AR, Vane JR. *Coronary vasoconstriction in vitro in the hearts of polyarthritic rats: effectiveness of in vivo treatment with the endothelin receptor antagonist SB209670.* Br. J. Pharmacol. 114, 1327-1328.

Larbre JP, Moore AR, Willoughby DA. *Cartilage destruction via the synovial fluid in rheumatoid arthritis.* J. Rheumatol. 22, 1438-1445.

Willis D, Moore AR, Gowland G, Willoughby DA. *OM-89 modulation of chronic inflammation: relevance to clinical use.* Br. J. Rheumatol. 34, 525-528.

Gowland G, Willis D, Moore AR, Willoughby DA. *OM-89 modulation of chronic inflammation: relevance to usage in human rheumatoid arthritis.* Rheumatology in Europe 24(S3), 186.

Willis D, Moore AR, Gowland G, Willoughby DA. *The effect of tolerance and sensitisation to the bacterial extract OM-89 on a polyarthritis in the rat.* Rheumatology in Europe 24(S3), 186.

Willis D, Gowland G, Moore AR, Willoughby DA. *Neonatal and adult tolerance to OM-89.* Inflammation Research 44(S3), S275.

Willis D, Frederik R, Moore AR, Willoughby DA. *Modulatory effect of heme oxygenase activity on sa model of acute inflammation in the rat.* Inflammation Research 44(S3), S252.

Willis D. Frederik R, Moore AR, Willoughby DA. *Activity and expression of heme oxygenase in two immune models of acute inflammation in the rat.* Inflammation Research 44(S3), S252.

Gowland G, Moore AR, Willis D, Willoughby DA. *Hyaluronan augments the actions of cyclosporin in avridine induced polyarthritis.* Inflammation Research 44(S3), S249.

Brown J, Willis D, Moore AR, Willoughby DA. *Apoptosis, heat shock protein 70kDa and tumour supressor p53 expression in a model of acute inflammation in the rat.* Inflammation Research 44(S3), S249.

Seed MP, Trancart MM, Moore AR, Azam Z, Prigent D, Willoughby DA. *Metalloproteinase (MMP) regulation and cartilage and cartilage matrix loss during granulomatous inflammation in elastase deficient Chediak-Higashi mice.* Inflammation Research 44(S3), S255.

Willis D, Moore AR, Gowland G, Willoughby DA. *Is tolerance induction a possible mode of action of OM-89?* 6th Interscience World Conference on Inflammation, Antirheumatics, Analgesics, Immunomodulators. Geneva 28-30 March 1995. abs 166.

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1994

Larbre JP, Moore AR, Da Silva JAP, Iwamura H, Willoughby DA. *Direct degradation of articular cartilage by rheumatoid synovialfluids: contribution of proteolytic enzymes.* Journal of Rheumatology In Press.

Tomlinson A, Appleton I, Moore AR, Gilroy DW, Willis D, Mitchell A, Willoughby DA. *Cyclooxygenase and nitric oxide syntllase isoforms in rat carrageenan-induced pleurisy.* Br. J. Pharmacol. In Press.

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Iwamura H, Moore AR, Willoughby DA. *Potentiation of activated neutrophil-induced chemiluminescence by the guanidine derivative EI*-130. British Journal of Pharmacology 107: 420P.

Larbre JP, Da Silva JAP, Hill R, Moore AR, Seed MP, Willoughby DA. *Etude des effets de l'interleukin 1 in vitro sur le cartilage humain en culture.* Revue du Rheumatisme 59: 688.

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The Inflammatory Response as a Modulator of Cartilage Breakdown - abstract of Ph.D thesis, 1991

An assay of rat femoral head cartilage was developed to allow changes in hyaline cartilage and mineralised cartilage to be differentiated. The assay was applied to a model of granuloma-induced cartilage breakdown in mice. Hyaline cartilage was degraded by a granulomatous reaction, but mineral content was increased. The model may have value in studying cartilage breakdown, detecting antirheumatic agents or investigating abnormal mineralisation processes.

The possible role of cartilage breakdown products in promoting the sustained migration of acute inflammatory cells into chronically inflamed joints was investigated. Cartilage glycosaminoglycans (GAGs) were tested for their effects on the locomotion of polymorphonuclear neutrophils (PMNs) *in vitro*. It was found that cartilage GAGs were chemotactic and that these activities were independent of complement activation. These agents also caused PMN accumulation *in vivo*.

A novel *in vitro* microassay allowed components of an inflammatory exudate to be incubated with cartilage matrix for periods upto 2 hours. PMNs and macrophages alone caused some cartilage degradation, but when the cells were stimulated with a variety of agents, only PMN-induced degradation was enhanced. Cartilage GAG loss by stimulated PMNs was inhibited by cell-free fluid exudate and serum but not by albumin at the same protein concentration.

Within 1 hour post stimulation, PMNs and macrophages were shown to produce reactive oxygen species (ROS), but only stimulated PMNs released increased amounts of lysosomal enzyme. Inhibitors/scavengers of ROS, when combined with stimulated PMNs, failed to protect cartilage from degradation. These results suggest that direct actions of proteolytic enzymes are of greater significance than ROS in cartilage breakdown.

Inflammatory cells, particularly stimulated PMNs, appear to mediate cartilage breakdown whereas fluid exudate appears to be inhibitory. The modulation of cartilage breakdown by inflammatory processes could have implications for anti inflammatory therapy in chronic inflammatory joint disease.

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AMELIORATION OF B16F10 MELANOMA LUNG METASTASIS IN MICE BY A COMBINATION THERAPY WITH INDOMETHACIN AND INTERLEUKIN 2

BY RANJIT S. PARHAR AND PEEYUSH K. LALA

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Metastasis remains the most formidable obstacle in cancer therapy. While there is reasonable evidence to suggest the existence of a natural cellular surveillance against the development of certain neoplasia (1), as well as the metastatic spread of tumor cells via circulation (2), such a surveillance often fails to contain the growth of most primary tumors or their metastatic dissemination. We have recently shown (3) in a number of murine tumor models, that the host NK cell activity declines rapidly during the development of transplanted or spontaneous tumors. This decline results from neither the disappearance of NK lineage cells (3) nor their maturation arrest (4), but from their rapid inactivation by the host-derived NK suppressor cells appearing in the lymphoid organs, as well as at the tumor site (4). They have been characterized as cells of the monocyte/macrophage class with phagocytic ability and bearing surface Mac-1. Their suppressor function is mediated by PG, and is abrogated in the presence of indomethacin, an inhibitor of PG synthesis (4). We have further noted that tumor-derived macrophages, or chemically pure PGE_2 can block a variety of lymphocyte responses in vitro: functional activation of NK cells, lectin (Con A)-induced lymphocyte proliferation and generation of killer cells, alloreactive proliferation of T cells and their subsequent differentiation into CTL, as well as the generation of lymphokine (rIL-2)-activated killer (LAK)[1] cells, the macrophage-mediated suppression being preventable in the presence of indomethacin (Lala, P. K., and R. S. Parhar, unpublished observations). These results suggest that the production of PG, particularly of the E series (PGE), may have pansuppressor effects against the activation of various classes of effector cells with tumoricidal potential in vivo in the tumor-bearing host. While the source of PGE has been identified as host macrophages in numerous tumor models in our study (4), certain tumors may also be capable of producing PGE (5). Furthermore, we have identified two important mechanisms of PGE_2-mediated blockade of T cell activation in the presence of alloantigens or a polyclonal mitogen Con A: a down regulation of IL-2-R development on the stimulated lymphocytes, and an inhibition of production of IL-2 by these cells; we saw no effect on the interaction between IL-2-R and IL-2, nor on the lytic function of CTL once generated (6).

This work was supported by the National Cancer Institute of Canada and the Medical Research Council of Canada.

[1] *Abbreviations used in this paper:* AGM1, asialo-GM-1; CCDF, cytotoxic cell differentiation factor; LAK, lymphokine-activated killer cell; LICC, lymphokine-induced cytotoxic cells.

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A down regulation of transferrin receptor development on T cells has also been documented (7). While the precise mechanism of PGE_2 action on NK lineage cells remains to be fully understood, a suppression of their activation (4) as well as their killer function (8, 9) has been documented.

We have recently examined the in vivo relevance of the PG-mediated suppression of natural host defence against cancer cells to their metastatic spread from the primary site (9). Using three C3H mammary carcinoma lines derived from a single spontaneous tumor, but varying in their lung metastasizing ability, we have observed that chronic indomethacin therapy shortly after a subcutaneous tumor transplantation produces the following effects: (*a*) a reduction in the growth of the primary tumor, (*b*) a prevention of lung metastasis, (*c*) a restoration of host NK activity, and (*d*) an abrogation of NK suppressor function of tumor-infiltrating macrophages (9). These results suggest that the PG-mediated NK suppression promotes tumor metastasis, which can be contained by indomethacin therapy. However, we find that lung metastasis from the primary tumors, once well established and advanced, can not be completely cured with indomethacin therapy when the therapy is provided at later intervals (Parhar, R. S., J. Nelson, and P. K. Lala, unpublished observations). Encouraged by the recent success of a combination therapy with IL-2 and LAK cells of established tumors and their metastases in mice (10–18) and man (18, 19), the present study was designed to test whether a combination therapy with indomethacin and IL-2 is superior to indomethacin alone or IL-2 alone in ameliorating established experimental metastasis of B16F10 melanoma cells. The rationale of this study was based on the contention that a relief of the endogenous suppression with indomethacin would allow a generation of sufficient levels of IL-2-R on the various effector lineage lymphocytes, which then can be stimulated further by the exogenous IL-2 provided over and above the endogenous IL-2 levels achieved by such therapy. Furthermore, we examined the characteristics of the splenic effector cells generated by such therapeutic measures in vivo, as revealed by their surface phenotype and cytocidal spectrum.

Materials and Methods

Mice. Young adult (8–10 wk old) C57BL/6 (henceforth called B6) mice (The Jackson Laboratory, Bar Harbor, ME) were used as hosts for the production of experimental metastasis.

Tumor. B16F10, a melanoma line with high lung metastasizing ability, produced initially by I. J. Fidler (20, 21), was obtained through the courtesy of A. Chambers of the Department of Radiation Oncology, The University of Western Ontario. This line, in our hands, does not produce any significant level of PGE_2 in vitro. Cells grown in tissue culture were injected intravenously at a high inoculum dose of 10^6 cells per mouse to produce experimental lung metastasis. Mice killed on day 5 all showed large numbers of well-established foci of microscopic metastases (micrometastases) in both lungs. Mice killed on day 10 all exhibited high numbers of visible (macro) metastases. Hosts usually died during week 4 after injection. Those killed on day 21 or autopsied at death showed confluent lung metastases of various sizes, and occasional melanotic foci in the liver. The experimental protocol was designed on the basis of this background information.

Experimental Protocol for Therapy. A total of 35 animals were injected intravenously via the tail vein with 10^6 melanoma cells. They were randomly assigned to seven groups of five animals per group. Five groups of experimental animals were subjected to the following therapeutic protocols: (Group 1) Indomethacin (Sigma Chemical Co., St. Louis, MO), at a concentration 14 μg/ml in drinking water, provided throughout the experi-

mental period from day 0 to day 21. Indomethacin was initially dissolved in absolute ethanol and then diluted in drinking water, resulting in a concentration of 0.2% ethanol, as reported earlier (9). Bottles were changed twice a week with freshly prepared materials. (Group 2) Indomethacin at a similar concentration provided from day 5 to day 21. (Group 3) Human rIL-2 (22, 23) (produced in *Escherichia coli*, lots 9A and LP9C, kindly supplied by the Cetus Corp., Emeryville, CA), 25,000 U per inoculum, injected intraperitoneally every 8 h for 5 d on days 10–14. (Group 4) Indomethacin, as given to group 1, plus rIL-2 as given to group 3. (Group 5) Indomethacin as given to group 2 plus rIL-2 as given to group 3. Two groups of control animals were used: the first group received vehicle alone used for dissolving indomethacin, i. e., 0.2% ethanol in drinking water throughout the experimental period (day 0–21); and the second group received the same drinking vehicle as above (day 0–21), plus intraperitoneal injections of the excipient control (solvent buffer for IL-2) every 8 h for 5 d (days 10–14).

Experiments were repeated for a second time to include group 1 as above in the control series, and groups 2 and 5 in the experimental series, using six mice per group. Since these essentially reproduced the results of the first series, the detailed results of the second series will not be presented.

Killing of Animals. All animals (both experimental and control groups) were killed on day 21, followed by a thorough visual examination of all internal organs for visible melanotic nodules, identifiable on the basis of the black melanin pigment. Lungs were removed and fixed in Bouin's Fixative for melanoma colony counts (24) and subsequent histological preparation. With this fixation procedure, black melanotic nodules stood out prominently on the light yellow background. Freshly isolated spleens were used to make single cell suspensions, free from red cells and debris (3), for use as effector cells in killer-cell assays against numerous tumor targets. For this purpose, cells were pooled from the animals in each group.

Killer-Cell Assay. A 4-h ^{51}Cr-release assay, as described earlier (3), was used to measure the percent specific cytotoxicity of spleen cells at various E/T ratios (12.5:1–100:1). The cytocidal spectrum was assessed by using the following tumor targets, all obtained from exponential phase growth in tissue culture and exhibiting >98% viability: YAC-1 lymphoma (a highly NK-sensitive line); B16F10 melanoma (a moderately NK-sensitive line in our hands), and thymic lymphoma 9705 (an NK-resistant line, kindly provided by Dr. F. P. H. Chan of the Department of Anatomy, The University of Western Ontario).

Phenotyping of Killer Cells. Killer cells were phenotyped for the presence of the surface markers Thy-1, Lyt-2, and asialo-GM-1 (AGM1) by using a complement-mediated cytotoxicity assay in the presence of appropriate antibodies. Monospecific anti-Thy-1.2 antibody (E Thy-1.2 of the National Institutes of Health [Bethesda, MD] catalogue, courtesy of Dr. J. G. Ray, NIH) and anti-Lyt-2 mAb (Becton Dickinson & Co., Sunnyvale, CA) were used at 1:20 dilution. Rabbit anti-AGM1 (Wako Chemicals, Dallas, TX) was used at 1:200 dilution. Rabbit complement (low tox, Cedarlane Laboratories, Hornby, Canada) was used at 1:20 dilution. These dilutions were based on control studies. Untreated spleen cells, or spleen cells treated with complement alone (controls), or with appropriate antibody (at 4°C for 45 min, followed by three washes), followed by complement (at 37°C for 45 min, followed by three washes) were used as effector populations. The E/T ratio was computed on the basis of the effector cell number before antibody or complement treatment so that the results could not be biased by an enrichment of a particular subset in the surviving effector population (25). All the treatment and the washes were carried out in the same tube to avoid any cell loss by transfer.

Statistical Evaluation. The significance of differences in the number of lung metastases between any two groups was determined by the Wilcoxon rank sum tests, using two-sided p values (26).

Results

Effects of Various Therapeutic Protocol on Experimental Lung Metastasis. Visible melanoma nodules detectable on day 21 after an intravenous inoculation of 10^6 melanoma cells reached a confluence in many of the control animals, the

TABLE I

Effects of Various Therapeutic Measures on Lung Colony Number

Protocol	Number of macroscopic melanoma nodules in the two lungs (day 21)			
	Range	Mean	Median	Percent reduction (median)
Control*	221–600+‡	418	473	0
	(297–600+)§	(471)	(473)	(0)
Indomethacin alone (days 0–21)	97–192	163	176	63
Indomethacin alone (days 5–21)	157–201	175	173	64
	(193–278)	(224)	(213)	(55)
IL-2 alone	102–174	146	152	68
Indomethacin (days 0–21) + IL-2	0–5‖	1	0	100
Indomethacin (days 5–21) + IL-2	0–9¶	2	0	100
	(0–17)	(9.5)	(9)	(98)

* Data in the two control groups were very similar and thus were pooled.
‡ The maximum number of countable nodules short of confluence was ~600.
‖ Only one animal showed five nodules.
¶ Only one animal showed nine nodules.
§ Data in parentheses represent results from the second series of experiments.

maximum scorable number in a pair of lungs being ~600. Both control groups provided very similar data and are thus pooled in Table I. Two of five animals in each control group also showed a small number of nodules on the surface of the liver, but on no other organs such as kidneys, spleen, intestine, mesentery, or reproductive organs. The two protocols of indomethacin administration alone (starting on day 0 or day 5) and the protocol of IL-2 therapy alone led to a substantial (all significant at $p \ll 0.01$) and very similar (63–68% median) reduction in the lung nodules as compared with the control animals. There was no significant ($p \gg 0.1$) difference between any two of these three experimental groups. A combination therapy with indomethacin plus IL-2, irrespective of the onset of indomethacin therapy (i. e., day 0 or 5) led to a complete freedom from visible melanotic foci on the lung surface in all animals, except one animal in each group that showed a small number of residual nodules also heterogeneous in size (Table I). The reduction of metastases with either protocol of combination therapy was highly significant ($p \ll 0.01$) compared with the control or either of the indomethacin groups, or the group receiving IL-2 alone. There was no significant difference ($p \gg 0.1$) in the results provided by the two protocols of combination therapy. None of the experimental animals exhibited any liver nodules. Representative photographs of the lungs are presented in Fig. 1.

A repetition of some of these experiments (second series) closely reproduced the results of the first series, as indicated by the data in parentheses in Table I.

A histological examination of the lungs that exhibited visible surface nodules also showed the presence of tumor cell foci in sections. Sections of nodule-free lungs of mice that had received a combination therapy were also free from microscopic foci of tumor cells. However, whether these lungs were totally devoid of tumor cells can only be answered on the basis of serial sections, which have not yet been performed. All experimental lungs exhibited visible levels of mononuclear cell infiltration. The infiltration was higher with IL-2 therapy than

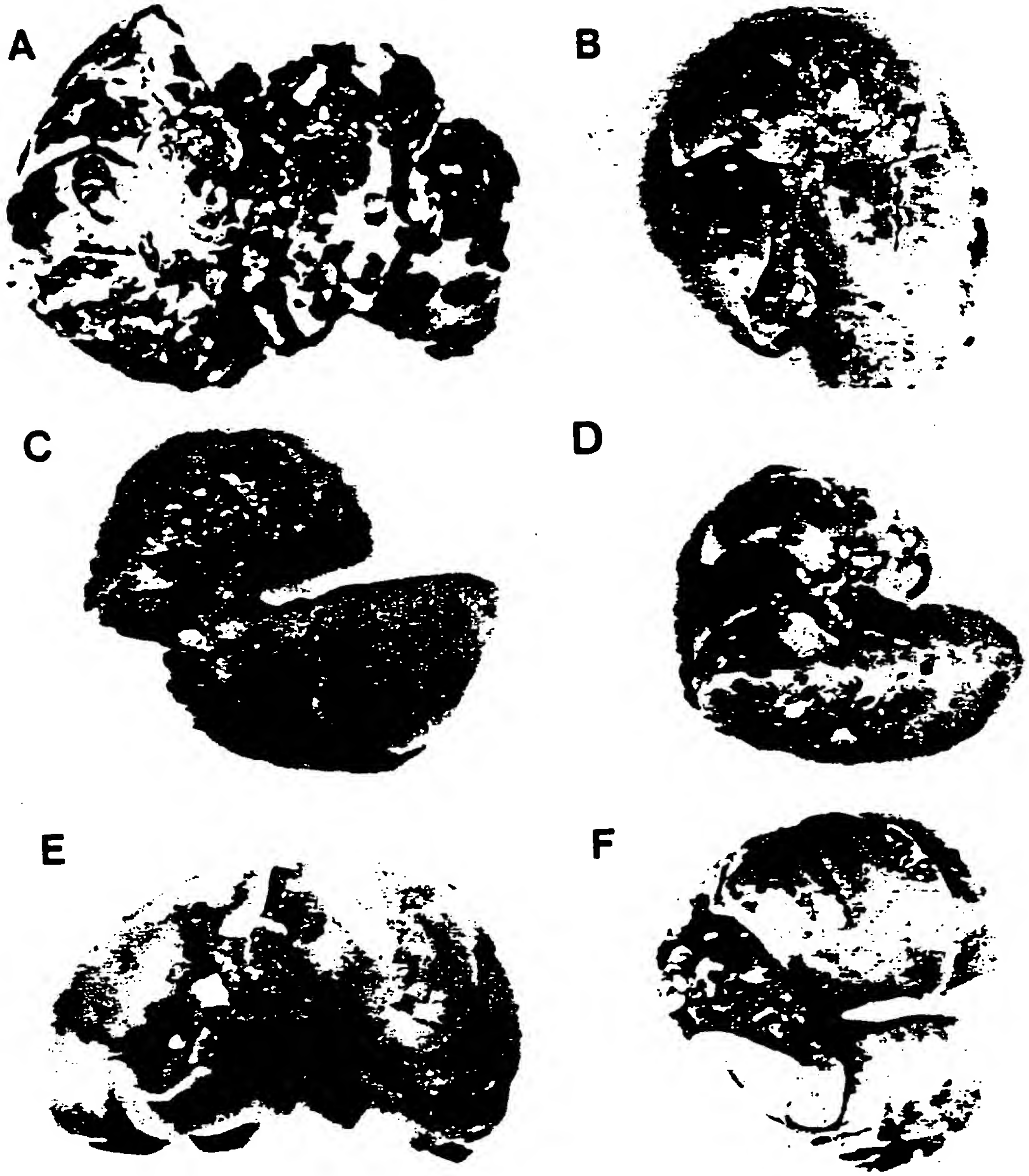

FIGURE 1. Representative photographs of the lungs from melanoma-inoculated mice. (*A*) Control mouse receiving 0.2% ethanol in drinking water from day 0 and excipient buffer (for dissolving IL-2) intraperitoneally on days 10–14. (*B*) Mouse receiving indomethacin alone from day 0. (*C*) Mouse receiving indomethacin alone from day 5. (*D*) Mouse receiving IL-2 alone on days 10–14. (*E*) Mouse receiving indomethacin from day 0 plus IL-2 on days 10–14. (*F*) Mouse receiving indomethacin from day 5 plus IL-2 on days 10–14.

with indomethacin therapy and was most marked with the combination therapy. In the latter case, alveoli in some areas of the lungs were totally replaced by young granulation tissue containing mononuclear cell exudate, possibly representing the sites of clearance of the original tumor foci. Representative histological pictures are presented in Fig. 2.

Cytocidal Spectrum and the Phenotype of Splenic Killer Cells Generated In Vivo. Results are only presented for the first series of experiments, since the second series closely reproduced the data as presented in the first series.

YAC-1 Target. Figs. 3 and 4 present the results of percent specific cytotoxicity

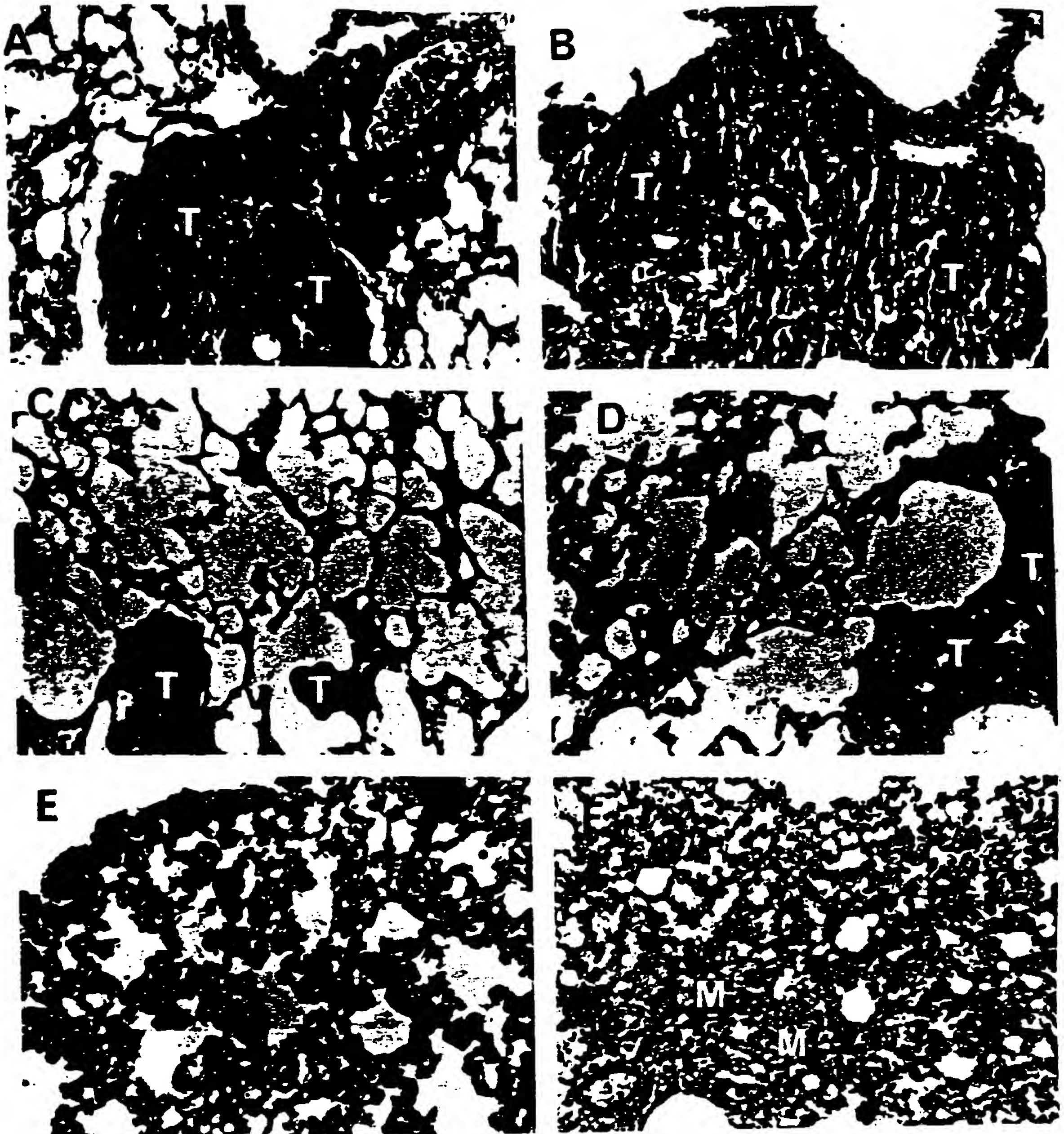

FIGURE 2. Representative photomicrographs (× 160) of H and E-stained sections of paraffin-embedded lungs of (*A*) mouse killed on day 5 after intravenous inoculation of 10^6 cells; (*B*) control mouse receiving 0.2% ethanol in drinking water from day 0 and excipient buffer (for dissolving IL-2) intraperitoneally on days 10–14; (*C*) mouse receiving IL-2 alone; (*D*) mouse receiving indomethacin from day 0 plus IL-2 on days 10–14; (*E*) mouse receiving indomethacin from day 0 plus IL-2 on days 10–14; (*F*) mouse receiving indomethacin from day 5 plus IL-2 on days 10–14. *T*, melanoma tumor nodules in *A–D*. Note variable degrees of interstitial mononuclear cell infiltration in *C–F*, more marked in *D* than in *C* and most marked in *E* and *F*. A large area of young granulation tissue represented by mononuclear cell exudate (indicated by *M*), replacing alveoli is shown in *F*. Such areas are encountered in both groups *E* and *F*, although not shown in *E*. They possibly represent the replacement sites of original melanoma nodules.

of normal spleen, spleen of control tumor-inoculated mice, or experimental tumor inoculated mice subjected to different therapeutic protocols. As expected, YAC-1 target was sensitive to lysis by normal B6 splenic effector cells that

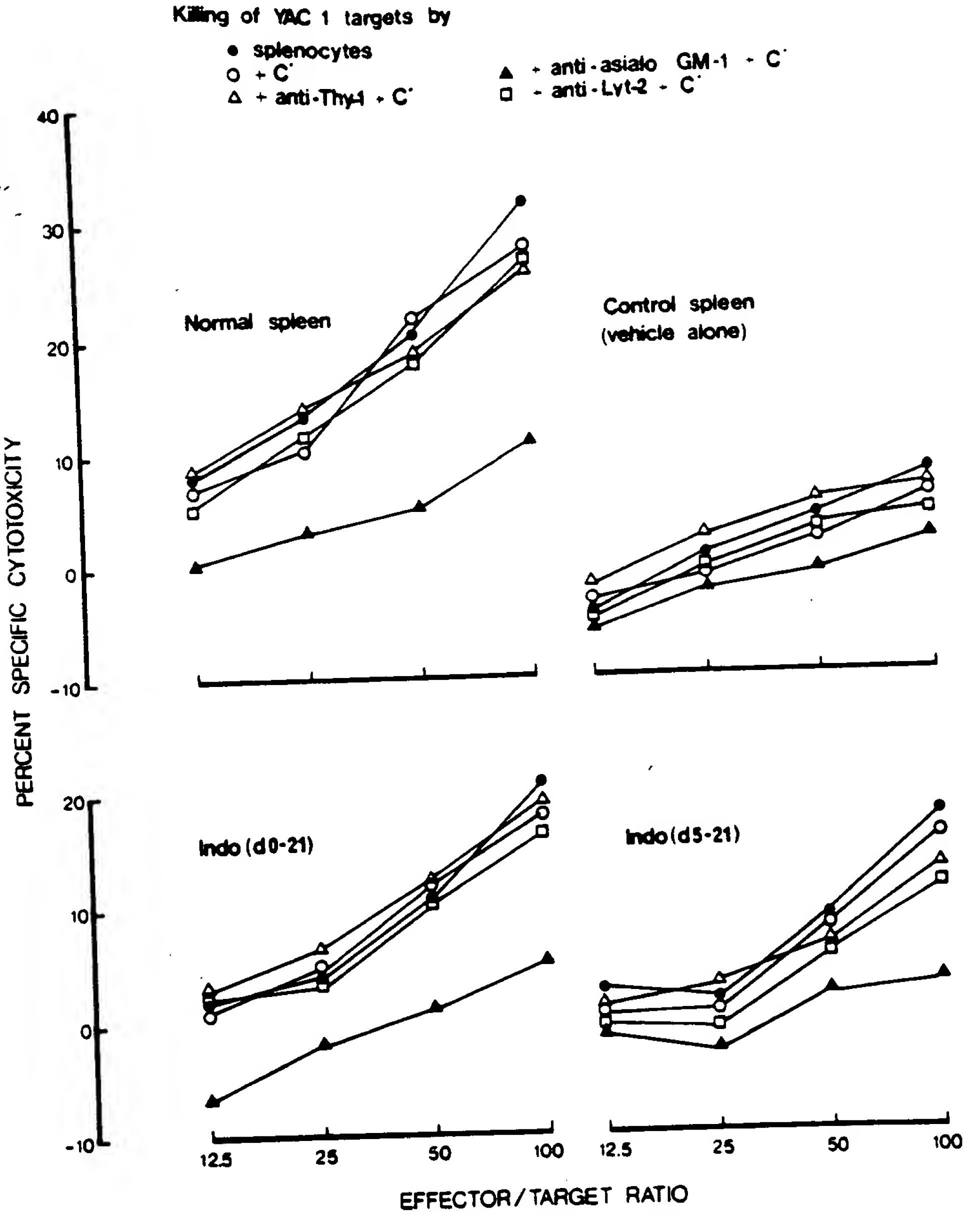

FIGURE 3. Anti YAC-1 cytotoxicity of splenic effector cells (subjected to various treatments) from normal healthy mice and tumor-inoculated mice treated with vehicle alone or indomethacin. Control (vehicle alone) group received 0.2% ethanol in drinking water from day 0. Control animals receiving the same treatment plus excipient buffer intraperitoneally (days 10–14) provided very similar results and are not presented. Effector cells were pooled from five mice in each group. Data represent means from triplicate wells with <5% variation. Indo, indomethacin.

expressed surface AGM1 but not Thy-1 or Lyt-2. Killer activity was reduced to near background levels in the spleen of control tumor inoculated groups. Treatment with indomethacin alone beginning on day 0 caused a substantial restoration of the killer activity measured on day 21, again AGM1$^+$, Thy-1$^-$ and Lyt-2$^-$ cells. Similar but somewhat less marked effects were produced by indomethacin therapy beginning on day 5 (Fig. 3). IL-2 therapy alone (Fig. 4) restored the killer function to above normal levels, and the effector cells were AGM1$^+$, Thy-1$^-$, and Lyt-2$^-$. Combination therapy with IL-2 plus indomethacin starting

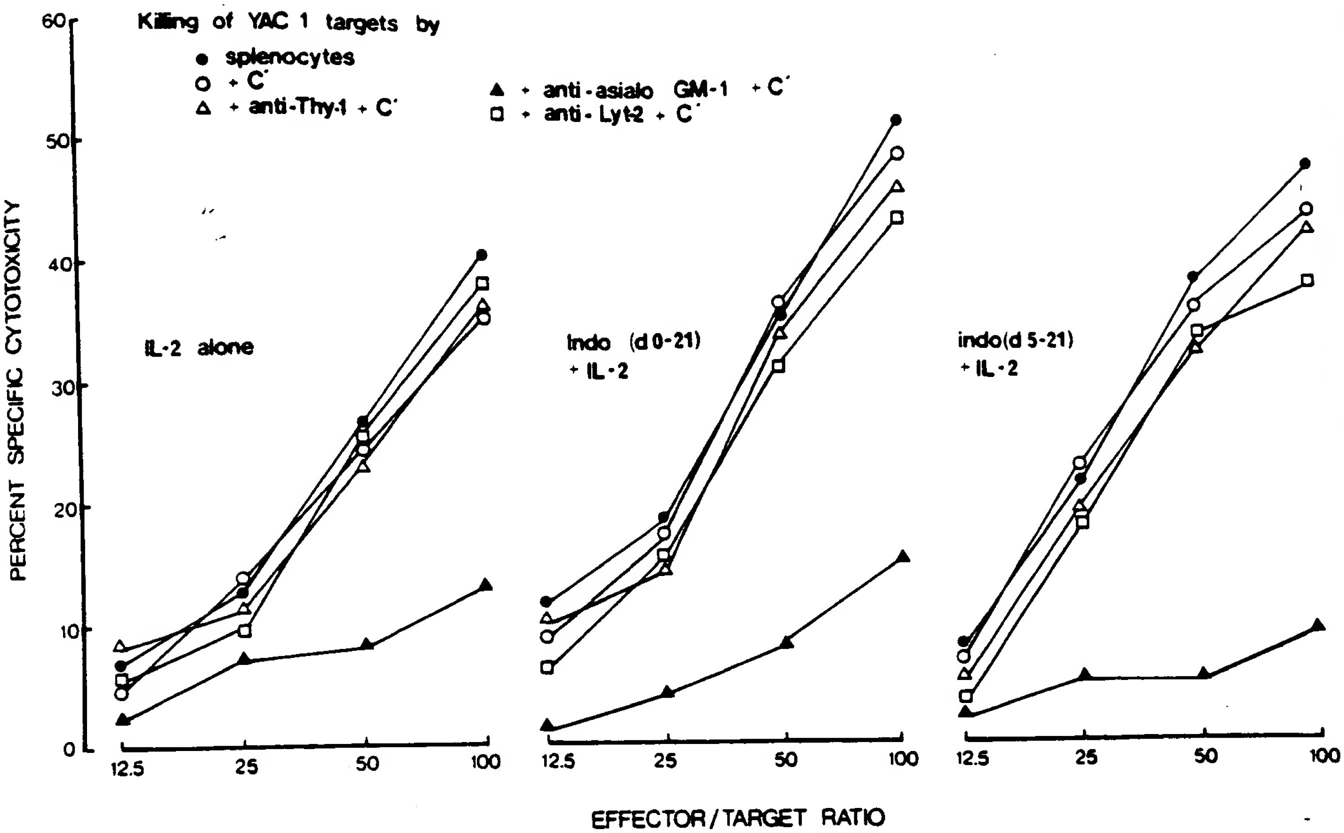

FIGURE 4. Anti YAC-1 cytotoxicity of splenocytes (subjected to various treatments) from tumor-inoculated animals treated with IL-2 alone or indomethacin plus IL-2. Effector cells were pooled from five mice in each group. Data represent means from triplicate wells with <5% variation. Indo, indomethacin.

either on day 0 or on day 5 stimulated the killer activity further and the killer cells were of the identical phenotype (Fig. 4).

B16F10 Target. B16F10 was found to be moderately sensitive to lysis by normal splenic effector cells, which also proved to be of the classical NK phenotype, i. e., $AGM1^+$, Thy-1^-, and Lyt-2^- (Fig. 5). Cytotoxic responses of this target to spleen cells from control tumor–inoculated mice or tumor–inoculated mice subjected to various therapeutic protocols were nearly identical to that of YAC-1 target, except for the fact that with indomethacin alone starting on day 0 or day 5, there was a complete restoration of killer function (Figs. 5 and 6). The killer cell phenotype, on all occasions, was again $AGM1^+$, Thy 1^-, Lyt 2^-.

Thymic Lymphoma 9705 Target. As shown by the poor cytolytic ability of normal splenic effector cells (Fig. 7), this tumor line is relatively NK resistant. Tumor bearing caused a further reduction of the splenic effector function, which was restored to control levels by indomethacin therapy starting on day 0 or day 5. IL-2 alone or IL-2 combined with indomethacin in either protocol caused an appreciable stimulation of killer activity above normal levels, the highest stimulation being provided with the combination therapy of IL-2 plus indomethacin beginning on day 0. In all cases, a nearly complete loss of effector function was only achieved with anti AGM1 + C′, indicating that the effector cells were again $AGM1^+$, Thy-1^-, and Lyt-2^-.

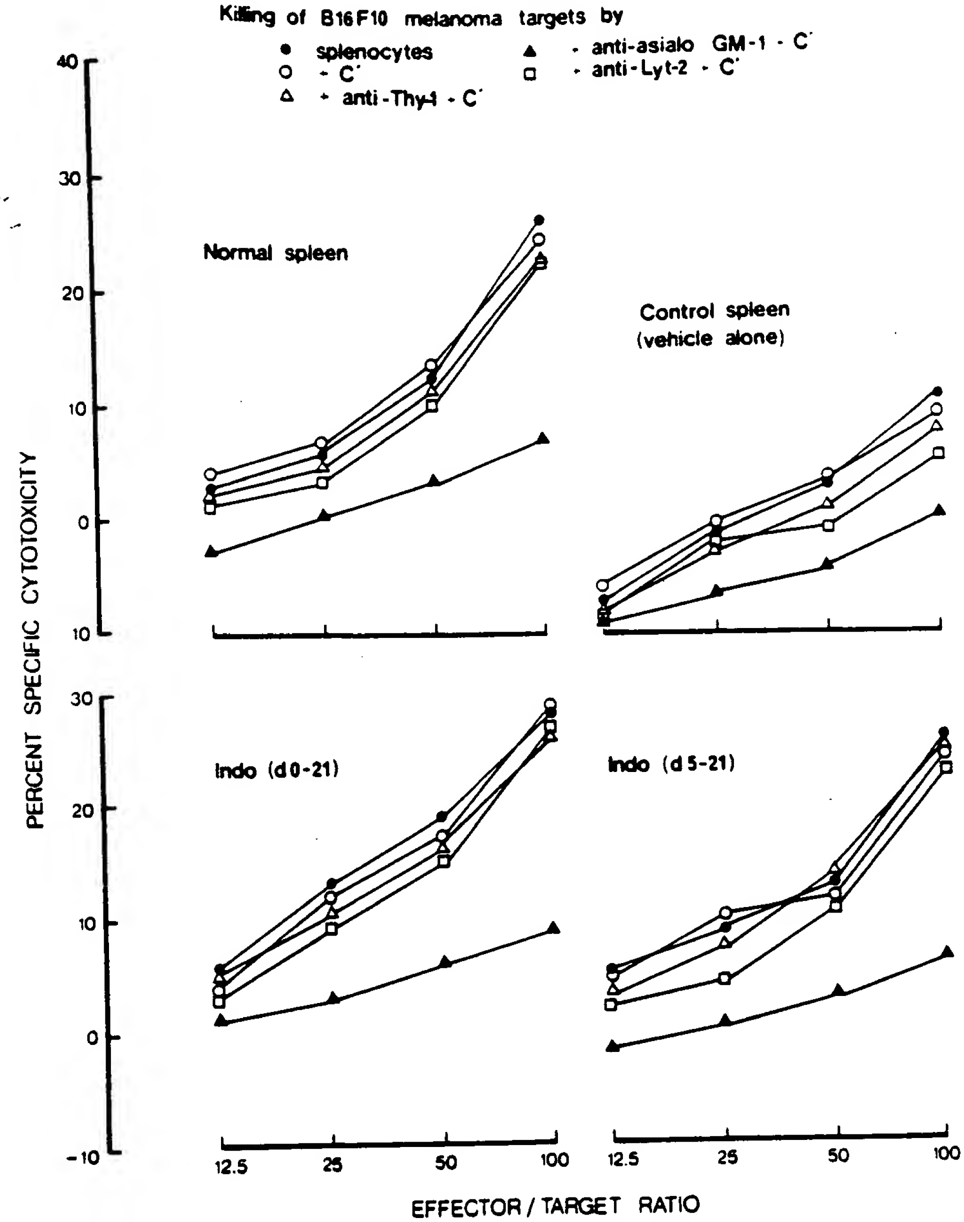

FIGURE 5. Anti-B16F10 cytotoxicity of splenocytes (subjected to various treatments) from normal healthy mice and tumor-inoculated mice treated with vehicle alone or indomethacin. Control (vehicle alone) group is the same as in Fig. 3. Those receiving, in addition, the excipient buffer intraperitoneally, provided nearly identical data (not presented). Effector cells were pooled from five mice in each group. Data represent means from triplicate wells with <5% variation. Indo, indomethacin.

Discussion

Chronic administration of indomethacin by the oral route (14 μg/ml of drinking water) or a 5-d regimen of intraperitoneal administration of 25,000 U IL-2 every 8 h led to a similar and substantial, but incomplete, containment of experimental lung metastasis produced by an intravenous injection of 10^6 B16F10 melanoma cells in B6 mice. Lack of any appreciable difference between the effects of the indomethacin regimen initiated on day 0 and day 5 can be explained by our earlier observations that tumor transplantation in mice causes

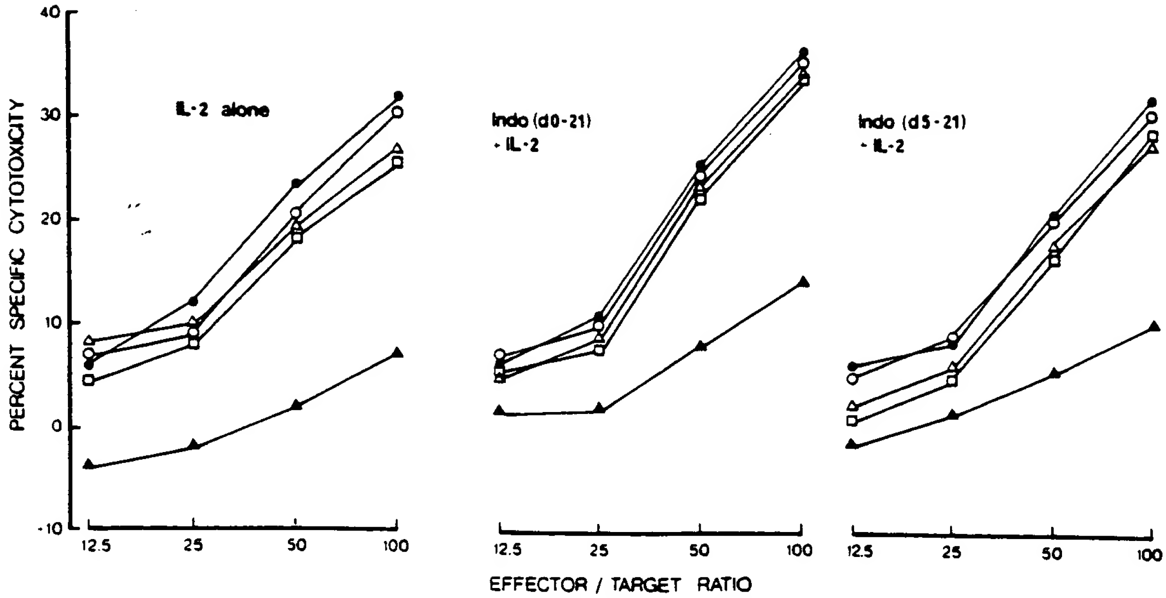

FIGURE 6. Anti-B16F10 cytotoxicity of splenocytes (subjected to various treatments) from tumor inoculated mice treated with IL-2 alone or indomethacin plus IL-2. Effector cells were pooled from five mice in each group. Data represent means from triplicate wells with <5% variation. Indo, indomethacin. Killing of B16F10 melanoma targets by: ●, splenocytes; ○, + C′; △, + anti-Thy-1 + C′; ▲, + anti-asialo GM-1 + C′; □, + anti-Lyt-2 + C′.

a transient and abortive stimulation of host NK activity, followed by a lasting suppression of this activity beginning on days 6–7 (3). Present results of indomethacin therapy alone are in agreement with reports from our own (9) as well as other laboratories (27–30) of the containing effects of this agent on tumor growth, and more importantly on the natural metastasis from the primary site. Similarly, the present results of IL-2 therapy alone, in spite of some differences in the protocol, are in essential agreement with those reported by Rosenberg's group in several murine models of metastasis, including other lines of B16 melanoma (10, 12–16, 18, 31). The inadequacy of indomethacin alone or IL-2 alone to affect a complete regression of established metastasis during the experimental period is explicable on the following grounds: (*a*) a relief of the endogenous PG-mediated suppression may permit a regeneration of IL-2 receptors on the effector lymphocytes as well as an improved synthesis of endogenous IL-2 (6), but the latter may still be inadequate for a maximal and sustained activation of the effector cells; (*b*) exogenous IL-2 alone may fail to cause an optimal stimulation, because of an inadequate generation of IL-2 receptors (6) on the effector lineage cells, if the endogenous suppression is not relieved. Such PG mediated suppression may, in addition, inhibit the production of other lymphokines such as IL-1 (32, 33) and cytotoxic cell differentiation factor (CCDF) (34–36), which are believed to be required for the lymphocyte activation/differentiation pathway. These possibilities are supported by our findings that chronic indomethacin treatment combined with a single round of IL-2 therapy led to a complete or nearly complete amelioration of experimental lung metastasis generated by a very high inoculum of B16F10 melanoma cells in a highly reproducible manner. The beneficial effects of this combination therapy seem to be comparable to the effects of combined IL-2 and LAK cell therapy of metastasis in the murine system (10–18). Furthermore, we have recently noted

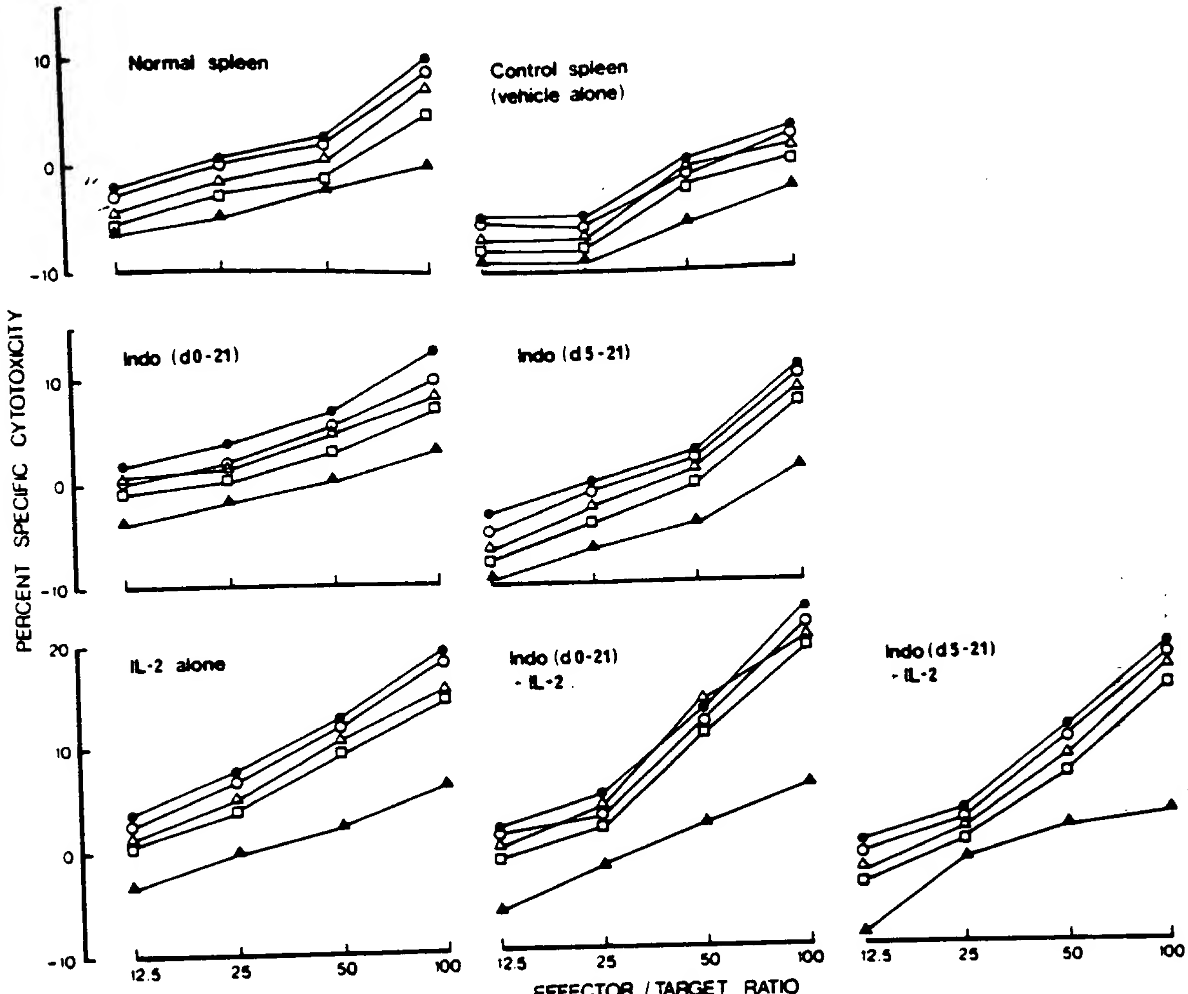

FIGURE 7. Cytotoxicity of splenocytes (subjected to various treatments) from normal healthy mice, and tumor-inoculated control and experimental mice (subjected to various therapeutic protocols) against the thymic lymphoma 9750. Control (vehicle alone) group is the same as in Fig. 3. Those receiving, in addition, the excipient buffer intraperitoneally, provided very similar results (not presented). Effector cells were pooled from five mice in each group. Data represent means from triplicate wells with <5% variation. Indo, indomethacin. Killing of thymoma targets by: ●, splenocytes; ○, + C′; △, + anti-Thy-1 + C′; ▲, anti-asialo GM1 + C′; □, anti-Lyt-2 + C′.

(Parhar, R. S., J. Nelson, and P. K. Lala, unpublished observations) that a combination of indomethacin and IL-2 can also cause a marked regression of spontaneous lung metastasis developing from a primary murine adenocarcinoma.

The effector cells generated in the host spleen by indomethacin alone, IL-2 alone, or a combination of indomethacin plus IL-2 in vivo, all expressed AGM1. This by itself cannot be considered as an exclusive NK cell marker, since AGM1 expression has also been reported (37) for cytotoxic T cells. However, an absence of Thy-1 or Lyt-2 on these cells indicates an NK-like phenotype. A superior functional activation of these effector cells was always achieved with the combination therapy, irrespective of the tumor target tested. With indomethacin alone, the cytolytic activity was essentially confined to the NK-sensitive targets YAC-1 and B16F10. Administration of IL-2 generated a broader spectrum of killer function extending also to the NK-resistant target thymic lymphoma 9705, the

killer activity being more marked with the combination therapy. Although this would suggest the generation of LAK-like cells in vivo, typical LAK cells generated in in vitro exhibit a different phenotype. They are Thy-1$^+$ and AGM1$^-$ and Lyt-2$^\pm$ (17, 18, 35; our unpublished observations). Another closely related effector cell class with a broad cytocidal spectrum generated in vitro from NK-like precursor cells requires the presence of IL-2 and a newly described lymphokine, CCDF, produced by macrophages in the presence of indomethacin (34–36). This cell class has been named lymphokine-induced cytotoxic cells (LICC), which are Thy-1$^+$, Lyt-2$^-$, and AGM1$^-$ (35). With the present combination therapy, one would expect that LICC and LAK cells should have been generated in vivo. Several possible explanations may be offered for the observed phenotypic discrepancy: (*a*) the effector cell phenotype may depend on its activation and differentiation status (36, 38) and may have been influenced in our case by a termination of the IL-2 therapy one week before mice were killed. In indomethacin-treated animals killed shortly after IL-2 therapy, splenic effector cells are found to be AGM1$^+$ and Thy-1$^\pm$ (Parhar, R. S., and P. K. Lala, unpublished observations); (*b*) an expression of Lyt-2 on the anti–tumor effector cells generated by IL-2 therapy in vivo may depend on the immunogenicity of the tumor concerned. The effector cells are Lyt-2$^+$ when tumors have detectable immunogenicity, but are Lyt-2$^-$ in the case of nonimmunogenic tumors (39), suggesting that IL-2 therapy may recruit both NK and T lineage cells; and (*c*) in the present study, the effector cells were sampled from the spleen and not from the tumor site i. e., the lungs, where a different subset may accumulate preferentially. Our preliminary (unpublished) studies reveal that AGM1$^+$, Thy-1$^+$ effector cells with high anti-B16F10 killer activity can be recovered from the lungs of the animals subjected to the combination therapy. The cytocidal spectrum and the phenotype of the various mononuclear cells infiltrating in situ, as well as the long term survival of mice placed under combination therapy, are currently under investigation.

Summary

Our earlier work revealed that PGE-mediated inactivation of NK cells in tumor-bearing mice by host macrophages promoted spontaneous lung metastasis that could be prevented or ameliorated by chronic indomethacin therapy. Since PGE was found to suppress the in vitro development and/or activation of a family of tumoricidal lymphocytes such as CTL, NK, and LAK cells by one or both of two mechanisms, that is to say, a down regulation of IL-2-R and an inhibition of IL-2 production, the present study tested whether a combined therapy with indomethacin and IL-2 was more effective than one with indomethacin or IL-2 alone in ameliorating established experimental lung metastasis. B6 mice injected intravenously with 10^6 highly metastatic B16F10 melanoma cells showed profuse micrometastases in the lungs by day 5, and macrometastases by day 10 which were confluent on day 21. Chronic indomethacin therapy by the oral route (14 μg/ml in drinking water) starting on day 0 or day 5, or a single round of IL-2 therapy (25,000 U rIL-2, every 8 h for 5 d on days 10–14) reduced the number of metastatic nodules by two-thirds (from a median of 473 in control mice receiving vehicles alone) by day 21. A single round of IL-2 as above, combined with either protocol of indomethacin therapy, completely or

nearly completely irradicated the lung metastases, corroborated by a histological examination. An evaluation of splenic killer cell activity measured with a 4-h ^{51}Cr-release assay against NK-sensitive YAC-1 lymphoma and B16F10 melanoma or NK-resistant thymic lymphoma 9705 targets revealed negligible activity in control tumor-bearing mice, and a good restoration of activity against NK-sensitive targets with either protocols of indomethacin therapy. IL-2 alone or a combination of IL-2 and indomethacin given by either protocol generated strong killer activity against all these targets, most marked with the combination therapy. Splenic killer cell phenotype in normal as well as all treated animals was ASGM1$^+$, Thy-1$^-$, and Lyt-2$^-$. The combination therapy resulted in the strongest mononuclear cell infiltration in the lungs, with areas of young granulation tissue suggestive of repair sites of original metastases.

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